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Is hybridization responsible for invasive growth of non-indigenous water-milfoils?

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Abstract

Heterosis, or hybrid vigor, has recently been proposed as a factor promoting invasive growth of some non-indigenous aquatic plant species, particularly those capable of spreading rapidly within and among lakes through clonal reproduction. We tested this hypothesis for variable-leaf water milfoil (*Myriophyllum heterophyllum*), a non-indigenous aquatic plant that has become a major management and conservation concern in New England. Using nuclear ribosomal DNA, we looked for F_1 hybrid populations of invasive *M. heterophyllum* in 25 New Hampshire (NH) lakes. In contrast to a previous study that found F_1 hybrid lineages of invasive *M. heterophyllum* in Connecticut, we did not find hybrids in our study lakes. This result has two implications: (1) pure lineages of *M. heterophyllum* are also capable of invasive growth, and (2) the distribution of invasive *M. heterophyllum* lineages (hybrid vs. pure) may be spatially structured across New England. We stress the importance of more detailed distributional and ecological studies for understanding the invasive potential of this species.

Introduction

Non-indigenous aquatic plants have large impacts on local biodiversity and ecosystem functioning (Ruiz et al. 1999). For example, the replacement of native *Spartina alterniflora* by non-native *Phragmites australis* is accompanied by changes in both soil physical properties (Windham and Lathrop 1999) and microbial community structure, which may in turn affect biogeochemical processes (Ravit et al. 2003). Similarly, the establishment and spread of non-indigenous Eurasian water-milfoil (*Myriophyllum spicatum*) alters light availability and reduces the amount of open water in the littoral zones of lake ecosystems, which can negatively affect the biodiversity of macrophyte, invertebrate, and fish communities (Madsen et al. 1991; Boylen et al. 1999; Buchan and Padilla 2000) In purely economic terms, the control costs for non-indigenous aquatic plants exceed 100 million dollars annually in the United States alone (Pimentel et al. 2000).

The biological basis for the vast ecological and economic impacts of many non-indigenous aquatic plants remains unknown. However, one hypothesis for the invasive growth of non-indigenous aquatic plants is heterosis, or hybrid vigor, resulting from hybridization with closely related species. In particular, heterosis may lead to the rapid spread of non-indigenous lineages when hybridization occurs between species that can propagate clonally, as is the case for many non-indigenous aquatic plants (e.g., Daehler and Strong 1997; Ayres et al. 2004; Moody and Les 2002).

The spread of non-indigenous variable-leaf water-milfoil, *Myriophyllum heterophyllum* (Haloragaceae), has become a major concern in New England. The geographic distribution of non-indigenous *M. heterophyllum* is less extensive than its invasive congener, *M. spicatum* (Eurasian water-milfoil), but is locally dominant when it establishes and is becoming more widespread in NH. As such, *M. heterophyllum* can be considered as a type IVb-V invader using the recent terminology proposed by Colautti and MacIsaac (2004) (hereafter referred to as "invasive growth" for simplicity). In addition, the ecological and economic impacts of *M. heterophyllum* throughout portions of New England rival those of *M. spicatum* elsewhere in North America. The rapid spread of *M. heterophyllum* over the past decade in NH has led to decreased native plant diversity and interference with lake recreational activity (A. Smagula, pers. comm.) and lake-front property values may decline by as much as 20–40% following colonization by *M. heterophyllum* (Halstead et al. 2003).

Recently, it has been hypothesized that heterosis may be responsible for invasive growth of *M. heterophyllum* (Moody and Les 2002). Using nuclear DNA sequences from the internal transcribed spacers (ITS), Moody and Les (2002) unequivocally demonstrated several occurrences of non-indigenous *M. heterophyllum* F_1 hybrid lineages in Connecticut (CT) ponds. Furthermore, they noted that populations of pure *M. heterophyllum* "rarely exhibited invasive characteristics, whereas the hybrids always did" (Moody and Les 2002). However, the spatial distribution of pure and hybrid *M. heterophyllum* lineages in New England is largely unknown.

We surveyed invasive *M. heterophyllum* populations in NH lakes to determine whether invasive populations were of hybrid origin. We expected to find F_1 hybrid *M. heterophyllum* lineages in our study lakes if heterosis determines invasive growth. On the other hand, the absence of hybrid lineages in our survey would indicate that (1) factors besides heterosis, such as environmental factors, can also contribute to invasive growth, and (2) invasive *M. heterophyllum* may consist of multiple genetic lineages.

Materials and methods

Sample collection and preparation

We used plant survey maps from the NH-DES to identify lakes with invasive populations of *M. heterophyllum*. We sampled 25 of these lakes to determine whether invasive populations of *M. heterophyllum* were composed of hybrid lineages in NH lakes. Tissue samples were taken by cutting an apical meristem from a single plant stalk and freeze-drying in a Labconco 77500 bench-top freeze dryer (−45 °C) for 48 h. We obtained one plant sample per lake for DNA analysis from the majority (23) of lakes. We assumed that vegetative reproduction in milfoils would result in many genetically identical plants within a lake, and therefore decided to sample as many lakes as possible to maximize the possibility of detecting hybrid populations. However, we also assessed within-lake genetic variability by obtaining multiple samples from different parts of two different lakes (Balch Pond, $n = 4$; Hopkinton Lake, $n = 2$).

DNA Analysis

We extracted DNA from freeze-dried samples using DNEasy Plant Mini Kits (Qiagen). We amplified the internal transcribed spacers 1 and 2 plus the intervening 5.8S ribosomal DNA subunit (hereafter referred to collectively as ITS) using the universal primers ITS1 and ITS4 (Soltis and Kuzoff 1995). PCR reactions consisted of the following: 2.5 μl buffer (GibCo), 1 μl $MgCl_2$ (2 mM), 2.5 μl of each primer, 2.5 μl dNTPs, 1 unit of Taq (GibCo), 2 μl template DNA filled to a final volume of 25 μl with sterile, distilled, and deionized water. Thermal cycling consisted of one cycle at 94 °C for 2 min followed by 25 cycles of: 94 °C, 1 min; 56 °C, 30 s; 72 °C, 1 min. A final extension at 72 °C for 8 min was followed by a hold at 4 °C. We ran PCR products on an agarose gel (~1.5%) to check for correct size and purity. We purified PCR products using the PCR Purification Kit (Qiagen).

Cycle sequencing was performed using ABI-Prism Big-Dye Terminator chemistry (Perkin Elmer) and sequences were run on an ABI 3100 automated DNA sequencer at the Dartmouth College Molecular Biology Core Facility. Sequences were edited using Sequencher (version

4.0.5) and aligned to GenBank accessions (AF513822-AF513850) of *Myriophyllum* spp. from Moody and Les (2002) using ClustalX (version 1.81, Thompson et al. 1997).

All sequencing reactions yielded "clean" DNA sequences without any double peaks as expected if there were F_1 hybrid genotypes. Thus, before any phylogenetic analysis, we were already aware that there were no hybrids in our samples. Nevertheless, we performed minimum evolution and maximum parsimony searches in MEGA (version 2, Kumar et al. 2001) to confirm that our DNA sequences were in fact most closely related to previously-published *M. heterophyllum* sequences available on GenBank. A Kimura two-parameter model of nucleotide substitution was used in the minimum evolution analysis. Statistical support for nodes in the phylogenetic analyses was determined through 1000 bootstrap replicates.

Results

We did not find any F_1 hybrid lineages of *M. heterophyllum* in our study lakes. Instead, we found a single ITS allele ("heterophyllum NH") in all 29 of our samples. This allele clearly grouped phylogenetically with *M. heterophyllum* Gen Bank accessions (Figure 1), indicating that these individuals are pure, not hybrid, *M. heterophyllum*. In fact, this allele only differed from *M. heterophyllum* GenBank samples from Connecticut and Minnesota by one nucleotide substitution in a total of 685 base pairs. We deposited the DNA sequence for this allele in GenBank (accession number AY817746).

Discussion

Heterosis has recently been identified as a potential explanation for the invasive growth of some non-indigenous plant species that can propagate via vegetative reproduction (Moody and Les 2002; Ayres et al. 2004). We tested this hypothesis for non-indigenous *M. heterophyllum* in NH lakes, but did not find F_1 hybrid genotypes in any of our samples, which comprised over half of all the known invasive *M. heterophyllum* populations in NH. The lack of hybrids in our study

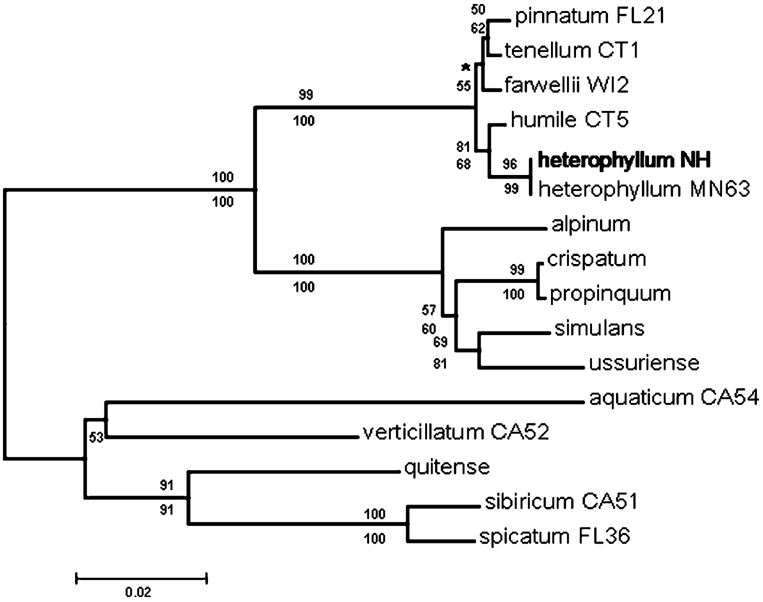

Figure 1. Phylogenetic relationships among the single ITS allele, "heterophyllum NH" (bold face) found in our *M. heterophyllum* samples and those available on GenBank (from Moody and Les 2002). We show only a single sample from each milfoil species available on GenBank for simplicity. Codes after sample names for GenBank sequences refer to the US postal codes of the states in which those samples originated. Numbers above and below nodes indicate bootstrap values for nodes from maximum parsimony and minimum evolution analyses, respectively.

demonstrates that heterosis is not required for invasive growth of *M. heterophyllum* populations; pure strains of *M. heterophyllum* are also invasive in many NH lakes.

Our results do not preclude a role for heterosis in the invasive growth of some *M. heterophyllum* populations. For example, hybrid *M. heterophyllum* from Moody and Les (2002) may have been more invasive than pure *M. heterophyllum* lineages in CT. Our results, however, demonstrate that hybridization is not common, at least in NH, and that heterosis is not the only mechanism leading to invasiveness. One explanation for our inability to detect hybrids is that these lineages are spreading northward but simply have not reached NH yet. In fact, some evidence already suggests this; the *M. heterophyllum* × *M. pinnatum* hybrids (Moody and Les 2002) presumably originated elsewhere and subsequently colonized CT lakes since *M. pinnatum* does not actually occur in CT. If this invasion scenario is true, then NH lakes may see a second wave of invasion by hybrid *M. heterophyllum* lineages. Thus, the invasion of *M. heterophyllum* in New England may be more complex than previously thought, involving multiple invasive lineages distributed across the landscape. Future studies should explore occurrence of hybrid and pure lineages in areas where *M. heterophyllum* is considered invasive and non-invasive.

We also recognize that hybrid lineages may occasionally occur in NH lakes, but simply went undetected because our sampling efforts placed more emphasis on among-lake instead of within-lake

Table 1. Sampling locations for our survey of invasive *M. heterophyllum* populations in NH lakes. Many of the study lakes have been treated with herbicides multiple times over the period of 1981–2003 to curtail invasive growth. Untreated lakes however also have invasive populations but have not been treated because of management priorities and regulations (A. Smagula, pers. comm.). Parentheses next to lake names refer to sampling locations within that lake.

Lake	Number of treatments	Method of Treatment	Evidence for Hybridity
Balch Pond (Woodman Dam)	1	Diquat	No
Balch Pond (Floating Island)	1	Diquat	No
Balch Pond (G)	1	Diquat	No
Balch Pond (Molson)	1	Diquat	No
Bixby Pond			No
Brindle Pond			No
Cheshire Pond			No
Crescent Lake	5	2,4-D, Diquat	No
Forest Lake	1	Diquat	No
Hill Top Pond			No
Hopkinton Lake (2 locations)	2	2,4-D, Diquat	No
Horseshoe Pond	1	2,4-D	No
Lees Pond	1	Diquat	No
Locke Lake	6	Endothall, Diquat	No
Melendy Pond			No
Northwood Lake	4	Diquat	No
Opeechee Park Pond			No
Lake Winnipesaukee (Paugus Bay)	2	Diquat	No
Pearly Pond			No
Phillips Pond			No
Potanipo Pond	1	Diquat	No
Squam Lake, Little	3	2,4-D, Diquat	No
Squam River			No
Sunapee Lake (Georges Mills)			No
Turkey Pond	3	2,4-D, Diquat	No
Turtle Pond	1	Diquat	No
Winnisquam Lake (Belmont)			No
Woodman Lake			No

sampling. That being said, we did not find any F_1 hybrid individuals in Hopkinton Lake or Balch Pond, which were sampled two and four times, respectively (see Table 1). Furthermore, although we may have failed to detect F_1 hybrid genotypes in an individual lake, the probability is much lower that we would fail to find any F_1 hybrids among our samples across 25 lakes if heterosis was the primary determinant of invasive growth.

Environmental factors often determine whether non-indigenous plant species grow invasively in new environments (Daehler 2003). In particular, invasive plants tend to outcompete native plants when nutrients and disturbance regimes are high relative to pre-invasion conditions (Daehler 2003). In the case of milfoil invasions, it is possible that increased nutrient inputs and lake disturbances arising from increased recreational use might facilitate both their spread and establishment. Dispersal by motorboats and boat trailers has been largely blamed for the spread of non-indigenous milfoils (Smith and Barko 1990) and preventative management efforts have subsequently focused on the establishment of laws that require removing plant debris from boats and trailers to prevent their spread. However, it is extremely likely that environmental conditions contribute to the development of invasive populations once they have arrived at a particular lake, regardless of the dispersal vector. Cultural eutrophication of lakes (see Lennon et al. 2003) and escape from natural enemies (Colautti et al. 2004) have been hypothesized to facilitate biological invasions for some non-indigenous species in lake ecosystems. Our results suggest that future research should focus on the role of environmental and ecological factors that may facilitate invasive growth of both pure and hybrid lineages of *M. heterophyllum*.

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